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BASIC INVESTIGATION

Effect of Lichong Decoction on expression of IGF-I and proliferating cell nuclear antigen mRNA in rat model of uterine leiomyoma

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Abstract

OBJECTIVE: To study the effect of Lichong Decoction (Lichong Decoction for strengthening anti-pathogenic *Qi* and eliminating blood stasis) on the expression of insulin-like growth factor-I (IGF-I) and proliferating cell nuclear antigen (PCNA) mRNA in a rat model of uterine leiomyoma.

METHODS: Fifty female Wistar rats were randomized into a normal control group, model group, Lichong Decoction group, Guizhifuling Capsule (Capsule containing Cassia Twig and Poria) group, and Mifepristone group. The uterine leiomyoma model was established by peritoneal injections of exogenous estrogen and progesterone hormone. The ultrastructural changes in cells of rat uterine tissues were observed with transmission electron microscopy, and the expression of IGF-I and PCNA mRNA was detected by real-time fluorescent quantitative PCR.

RESULTS: Following treatment, cells in the Lichong Decoction group appeared to be arranged normally, the cellular morphology were almost in a normal state, hyperplasia and hypertrophy of the chondriosome was reduced, collagen fibers were arranged in a regular manner, without obvious hyperplasia, and the expression of IGF-I and PCNA mRNA was significantly decreased compared with the model group (P<0.01).

CONCLUSIONS: The effect of Lichong Decoction on uterine leiomyoma is related to its function in reducing the expression of IGF-I and PCNA mRNA.

Key words: Leiomyoma; Insulin-like growth factor I; Proliferating cell nuclear antigen; Lichong Decoction

INTRODUCTION

Uterine leiomyoma, which is a common disease of the female reproductive system, has a great influence on women's health and quality of life. Studies have shown an association between uterine leiomyoma and excessive proliferation of uterine smooth muscle cells.[1,2] In addition, the expression of insulin-like growth factor-I (IGF-I) and proliferating cell nuclear antigen (PCNA) has been shown to affect cell proliferation, thus the molecular basis of uterine leiomyoma development is related to the overexpression of IGF-I and PCNA.[3,4]

Through the establishment of a rat model of uterine fibroids, using the real-time quantitative PCR technique, this study observed the effect of Lichong Decoc-

tion, which has the function of strengthening anti-pathogenic *Qi* and eliminating blood stasis, on the expression of IGF-I and proliferating cell nuclear antigen (PCNA) mRNA in a rat model of uterine leiomyoma. The study also explored the molecular mechanisms of this Chinese medicine in strengthening anti-pathogenic *Qi* and eliminating blood stasis to inhibit uterine fibroids for the purpose of providing a scientific experimental basis for clinical treatment.

MATERIALS AND METHODS

Reagents and instruments

Fermentas K1622 RT-PCR Kit (ABI, USA); SYBR Green PCR Master Mix (ABI, USA); TRIZOL reagent (Invitrogen life technologies, USA); PRISM 5700 fluorescent quantitative PCR (ABI, USA); DU-600 ultraviolet spectrophotometer (BECKMAN, USA); High speed refrigerated centrifuge (BECKMAN, USA), JEM-1230 transmission electron microscope (JEOL, JAPAN).

Animals

Fifty healthy, non-pregnant, adult female Wistar rats, weighing 220 ± 20 g, of SPF grade, were purchased from the Academy of Military Medical Sciences, certified SCXK (Army) 2007-0004. Rats were bred in SPF animal rooms of the Ministry of Experimental Animals, Capital Medical University, with normal light conditions and free access to food and water, and were housed at room temperature (18°C-22°C).

Drugs

Estradiol Benzoate Injection: Tianjin Jin Yao Amino Acid Co., Ltd., Product batch number: 0703121, Specification: 1 mL:1 mg.

Progesterone Injection: Shanghai General Pharmaceutical Co., Ltd., Product batch number: 070902, Specification: 1 mL:20 mg.

Lichong Decoction from the *Yixue Zhong Zhong Can Xi Lu* (Records of Traditional Chinese and Western Medicine in Combination) as was written by the famous doctor Xichun Zhang of Qing Dynasty. The quantity of each medicine was converted from the original to the following prescription: raw Huangqi (Radix Astragali seu Hedysari) 9 g, Dangshen (Radix Codonopsis Pilosulae) 6 g, Baizhu (Rhizoma Atractylodis Macrocephalae) 6 g, raw Shanyao (Rhizoma Dioscoreae) 15 g, Tianhuafen (Radix Trichosanthis) 12 g, Zhimu (Rhizoma Anemarrhenae) 12 g, Sanleng (Rhizoma Sparganii) 9 g, Ezhu (Rhizoma Zedoariae) 9 g, raw Jineijin (Endothelium Corneum Gigeriae Galli) 9 g. The Chinese medicines were purchased from Beijing Tong Ren Tang Herbal Pieces Limited Liability Company. The medicines were first processed by water extraction twice, and then the preparation was concentrated to 3.30 g·mL^{-1}. The daily dose for one rat was 22 g·kg^{-1}. The prepared concentration was kept in low temperature (0°C-4°C), and shaken well before use.

Guizhifuling Capsule (Capsule containing Cassia Twig and Poria): Jiangsu Kangyuan Pharmaceutical Co., Ltd. Product batch number: 080305. The medicines in the capsules were poured out and dissolved in distilled water to make a final concentration of 0.10 g·mL^{-1}. The daily dose for one rat was 0.70 g·kg^{-1}.

Mifepristone (Houdingnuo): Zhejiang Xianju Pharmaceutical Co., Ltd. Product batch number: 080501. The drug was dissolved in distilled water to make a final concentration of 0.94 mg·mL^{-1}. The daily dose for one rat was 6.25 mg·kg^{-1}.

Modeling and drug administration

Experimental rats were fed normally for a week. After their adaptation, they were divided into groups according to the random number table for model building, the normal control group (n=10 rats), and the model group (n=40 rats). Rats from the two groups were caged separately and allowed to drink water and eat freely. The model group was injected intraperitoneally with estradiol benzoate(0.5 mg·kg^{-1}), once a day for 25 days in succession, and then injected intraperitoneally with progesterone (4 mg·kg^{-1})once a day for 5 days in succession. After modeling, the rat models were subdivided according to the random number table into model control group, Lichong Decoction group, Guizhifuling Capsule group, and Mifepristone group. Gavage was given to the rats in each treatment group, once a day, and the dosage for each rat was equal to two times of that for an adult person. The normal and model control groups were given gavage with an equal amount of distilled water every day, for 4 weeks in succession. The rats were weighed once a week. Observations on hair color and spontaneous activities of rats was monitored. After drug administration, the rats were made to fast for 12 h overnight without any limitation on water intake, and then weighed. Intraperitoneal anesthesia was applied with 30% (v/v) chloral hydrate (3 mL·kg^{-1}) and the uterus of each rat was removed for testing.

Cellular ultrastructure

Uterine tissue 1 cm above the uterine cornu was put into glutaraldehyde liquid, immediately cut into 1 mm^3 pieces, fixed, washed with the 2% (v/v) PBS, and embedded for sectioning. Changes in the cellular ultrastructure of tissue were observed using transmission electron microscopy.

Expression of IGF-I and PCNA mRNA

Using the real-time PCR, the influence of Lichong Decoction on the expression of IGF-I and PCNA mRNA was tested. TIRZOL (1 mL) was added to uterine samples (50 mg-100 mg) and RNA was extracted. Primers were designed using Primer Expression 3.0 (Table 1).

Sample RNA was reversely transcribed to cDNA using the Fermentas K1622 RT-PCR Kit. The reaction con-

Table 1 The primer sequences

Gene		Sequences
β-actin	up	5' gagaccttcaacaccccagcc 3'
	down	5' aatgtcacgcacgatttccc 3'
IGF-I	up	5' acatctcttctacctggcactct 3'
	down	5' aagcaacactcatccacaatg 3'
PCNA	up	5' caatttctagcaacgcctaagat3'
	down	5' aagaggaagctgtgtccatagag3'

Notes: IGF-I: insulin-like growth factor-I; PCNA:proliferating cell nuclear antigen.

ditions for real-time PCR amplification and testing were: 95°C for 5 min; 95°C for 30 s; 53°C for 30 s; 72°C for 50 s, 40 cycles; 72°C for 8 min. After the reaction, sample CT values were determined.

Statistical analysis

Data are shown as the mean ± standard deviation. The data were analyzed with SPSS13.0. If data were normally distributed, a one-way ANOVA was used to analyze differences among groups. When there was homogeneity of variance, the LSD test was adopted. If not, a Tambane's T2 test was performed. If data were not normally distributed, the Kruskal-Wallis H test was performed. $P<0.05$ was regarded as statistically significant.

RESULTS

Transmission electron microscopy

Normal control group: Uterine smooth muscle cells were in an ordered spindle shape. Around the cells, collagen fibers arranged regularly. The cellular organelles in the cytoplasm were normal (Figure 1A).

Model control group: Fibroid cells increased in size, the nucleus increased in volume, and some were observed to be malformed. Cells were rich in actin microfilament cables. Mitochondria were hypertrophied and hyperplasia was evident. The rough endoplasmic reticulum expanded and the number of free ribosomes increased. Around the cells, collagen fibers were hyperplastic, disordered, and irregular. These results indicated that model establishment was successful (Figure 1B).

Lichong Decoction group: Cells appeared ordered, and organelles were almost normal. The presence of actin microfilament cables was reduced when compared to the normal control group. Mitochondria were less hypertrophied. Collagen fibers were arranged relatively regularly. Hyperplasia was not obvious (Figure 1C).

Guizhifuling Capsule group: Cells were observed to have slight hyperplasia. The number of cellular organelles decreased. Some mitochondria appeared empty with bubble-like changes. The rough endoplasmic reticulum appeared to expand, but not to the same extent as the model control group. Collagen fibers were irregular and slightly hyperplastic (Figure 1D).

Mifepristone group: Cellular hyperplasia was reduced and the cell was rich in cellular organelles. There were some hyperplastic and irregular collagen fibers surrounding cells (Figure 1E).

Influence of Lichong Decoction on the expression of IGF-I and PCNA mRNA

Amplified PCR products: results: β-actin (264bp), IGF-I (201bp), and PCNA (231bp) Bands were bright and visible (Figure 2).

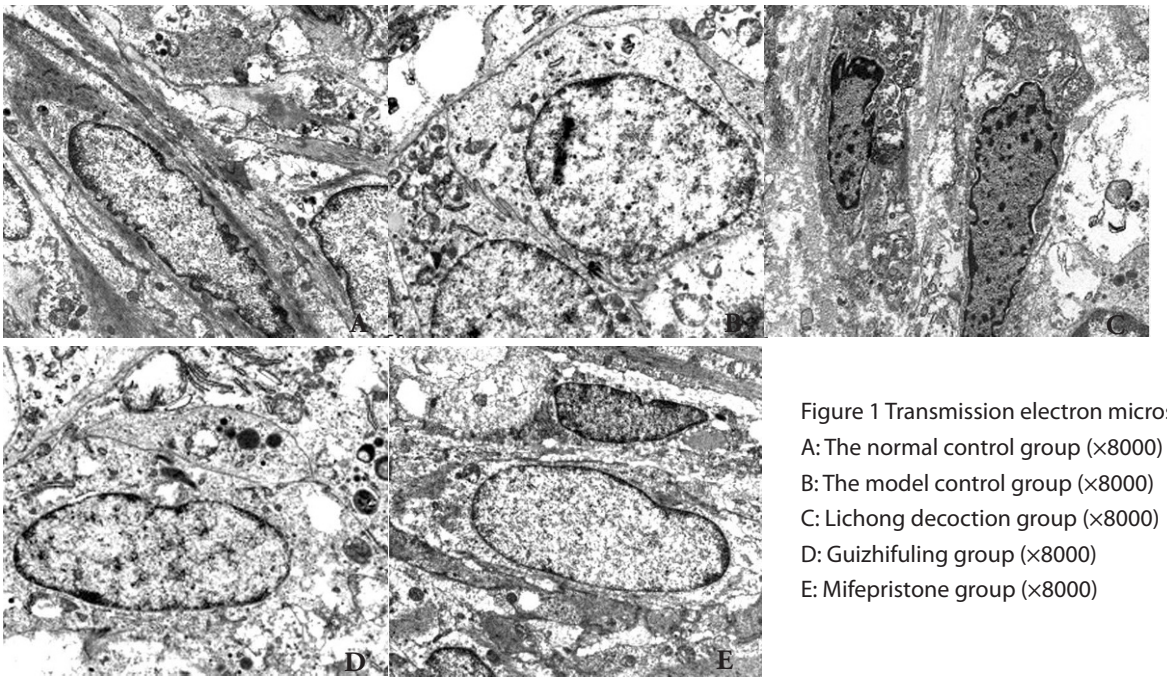

Figure 1 Transmission electron microscopy
A: The normal control group (×8000)
B: The model control group (×8000)
C: Lichong decoction group (×8000)
D: Guizhifuling group (×8000)
E: Mifepristone group (×8000)

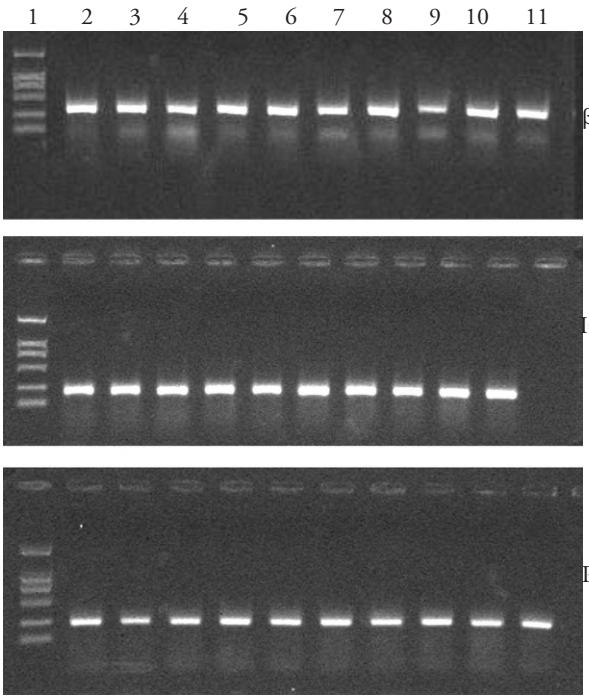

Figure 2 Amplified PCR products
IGF-I: insulin-like growth factor-I; PCNA:proliferating cell nuclear antigen; From left to right: 1, Marker; 2 and 3, Model control group; 4 and 5, Lichong Decoction group; 6 and 7, Guizhi Fuling group; 8 and 9, Mifepristone group; 10 and 11, Normal control group.

IGF-I and PCNA mRNA expression

Compared with the normal control group, the level of IGF-I and PCNA mRNA expression in the model group increased significantly (P<0.01). Compared with the model group, the level of IGF-I and PCNA mRNA expression in the Lichong Decoction group and Guizhifuling Capsule group decreased significantly (P< 0.01); and the level of IGF-I mRNA expression in the Mifepristone group decreased significantly (P<0.01), while PCNA mRNA expression was without statistical significance (Table 2).

Table 2 IGF-I and PCNA mRNA expression in each group (n=10, $\bar{x}\pm s$)

Group	IGF-I	PCNA
Normal control	0.54±0.06[a]	0.28±0.05[a]
Model	1.06±0.08	0.68±0.06
Li Chong Decoction	0.69±0.04[a]	0.32±0.07[a]
Guizhi Fuling Capsule	0.68±0.05[a]	0.39±0.04[a]
Mifepristone	0.78±0.06[a]	0.72±0.07

Notes: IGF-I: insulin-like growth factor-I; PCNA:proliferating cell nuclear antigen; Compared to model group, [a]P<0.01.

DISCUSSION

Uterine leiomyoma, the incidence of which is rising every year, is a commonly seen benign tumor of the female reproductive organs. It is one of the major reasons for hysterectomies and greatly influences women's physical and psychological health. The pathogenesis of uterine leiomyomas is not clear. Modern medicine suggests it is related to environmental factors, gene mutations, progestational hormones, growth factors, and changes in the extracellular matrix.[5-7] Modern medical treatments include surgery and hormone therapy. However, these treatments have a great influence on a woman's quality of life and have side effects, so most sufferers refuse to use them. Traditional Chinese Medicine has its own unique advantages in treating early-stage uterine leiomyomas, including good outcomes and few side effects. Therefore, it is necessary to investigate the effect of Traditional Chinese Medicine on uterine leiomyomas.

Uterine leiomyoma falls into the category of an "abdominal mass" in TCM according to its clinical manifestations. *Jing-yue Quan Shu and Fu Ren Gui* (The Complete Works of Jingyue Zhang – Woman Rules) state that "Long-term stasis developing into lumps is only a symptom observed in women. The symptoms may appear during menstruation or postpartum because of raw or cold food, invasion of cold, or anger damaging the liver, thus, *Qi* stagnation results in blood stasis; or long-term worries damage the spleen, leading to *Qi* deficiency, thus, causing blood stasis; or extreme physical weakness from exhaustion causes *Qi* deficiency, thus, causing blood stasis. The tumor is the final presentation of blood stasis". This statement points out that "deficiency" and "blood stasis" are the basic pathogenesis of uterine leiomyoma.

Jiaozhu Furen Liangfang (Collated and Annotated Effective Prescriptions for Women) states: "In general, dyspepsia and lumps are usually caused by excess pathogens during duration of deficiency in anti-pathogenic *Qi*. Reinforcing the anti-pathogenic *Qi* and removing excess pathogens makes the lumps dissolve automatically." Reinforcing the anti-pathogenic *Qi* to remove the excess pathogens, that is blood stasis, is the treating principle for uterine leiomyoma.

Lichong Decoction from the Records of Traditional Chinese and Western Medicine in Combination written by the famous doctor Xichun Zhang in the Qing Dynasty advocates reinforcing anti-pathogenic *Qi* to remove blood stasis, which both strengthens and elimi-

nates at the same time. In the prescription, raw Huangqi (Radix Astragali seu Hedysari), Dangshen (Radix Codonopsis Pilosulae), Baizhu (Rhizoma Atractylodis Macrocephalae), and raw Shanyao (Rhizoma Dioscoreae) function to reinforce *Qi* and strengthen the spleen; Sanleng (Rhizoma Sparganii), Ezhu (Rhizoma Zedoariae), and raw Jineinin (Endothelium Corneum Gigeriae Galli) are effective in dissolving lumps; Tianhuafen (Radix Trichosanthis) is used to replenish *Yin* and remove toxins; and Zhimu (Rhizoma Anemarrhenae) acts to cool the heat of Huangqi and Dang shen and supplement the kidney with water. Collectively, this prescription functions to reinforce *Qi* without disturbing the removal of stasis, and eliminates pathogens without damaging anti-pathogenic *Qi*.

During our early studies, we examined the effect of Lichong Decoction on the expression of related genes that control the death of cells and found that it could induce the death of myoma cells.[3] In order to further study the functions of Lichong Decoction, we monitored its effect on IGF-I and PCNA mRNA expression to understand its mechanism of action at the molecular level.

The complex pathogenesis of uterine fibroids is related to the abnormal expression of proliferation-related genes of uterine smooth muscle cells. IGF-I, containing 70 amino acids, is a peptide that has cell differentiation and proliferation functions, and insulin-like metabolism. This peptide has intense mitotic activity in normal cells and tumor cells.[9,10] IGF-I is also a common growth factor in the endocrine, paracrine, and autocrine systems, and regulates the proliferation and apoptosis in target organs to stimulate the growth of normal and tumor cells. Studies have shown that IGF-I can promote the mitosis of cells, and stimulate specifically the proliferation of uterine smooth muscles, thus, being closely associated with the development of uterine leiomyoma.[11,12]

PCNA is a coenzyme of DNA polymerase that is directly involved in the synthesis of DNA in the nucleus and is closely related to cell proliferative activity. PCNA is a highly sensitive and accurate indicator of cell proliferation.[13,14] PCNA is only synthesized and expressed in proliferating cells. Its synthesis reaches a peak in the S phase of the cell cycle, while in the G/M phase it decreases, so its content reflects cell proliferative activity.[15]

The results of this study show that compared with the normal control group, the level of IGF-I and PCNA mRNA expression in the model group increased significantly (P<0.01), indicating that high expression of IGF-I and PCNA mRNA is the molecular basis of uterine leiomyoma. When comparing the Lichong Decoction group with the model control group, our findings revealed that IGF-I and PCNA mRNA expression decreased significantly (P<0.01). After the treatment with Lichong Decoction, ultrastructural observations following transmission electron microscopy revealed that cells were arranged normally, cellular organelles were almost normal, actin microfilaments were reduced, mitochondria were less hypertrophied, and hyperplasia was not obvious. These results indicate that Lichong Decoction was effective on uterine leiomyoma, the mechanism of which is related to the reduction of IGF-I and PCNA mRNA expression. Further studies are needed to reveal the molecular mechanisms of Lichong Decoction in uterine leiomyomas.

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